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During the last 12-months, most work proposed under Specific Aim 1 was substantially completed. [*Aim 1;* aromatase mRNA translational efficiency will be determined by monitoring its distribution in both translationally active polysomes and translationally inactive monosome or messenger ribonucleotide particles (mRNPs). This approach will enable us to establish if promoter switching allows a given aromatase mRNA species to be translated more efficiently, thereby resulting in higher amounts of aromatase protein under these circumstances. Cellular amounts of aromatase mRNA–total and promoter-specific–will be determined by competitive reverse transcriptase polymerase chain reaction (RT-PCR)]. A murine cell-culture model of fibroblast-to-adipocyte differentiation was validated and used to demonstrate aromatase gene promoter-switching. Validation of a human cell culture model to be used in further studies is nearly complete. The pace of progress was adversely affected by the loss of a technician who departed in February 2004 to relocate out of state. Efforts to find a suitably qualified replacement were unsuccessful until October 2004. Completion of work proposed under Specific Aim 1, and Aims 2 and 3 during the current no-cost extension period will be greatly facilitated by the addition of a technician who has already been engaged to start work on November 29, 2004.

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Introduction

This project is based on evidence that in postmenopausal hormone-dependent breast cancer, *in situ* over-expression of aromatase (P450arom; the product of the CYP19 gene) by stromal cells results in increased local estrogen levels, which stimulate and/or support the proliferation of malignant breast epithelial cells (Green, 1990; Lippman et al., 1986). Conversely, estrogen deprivation induced by aromatase inhibitors is an effective treatment in some breast cancer patients (Goss and Strasser, 2001). Despite evidence supporting the importance of aromatase in initiation and development of breast cancer, knowledge about the regulation of aromatase expression is limited. Several distinct aromatase mRNA species have been found in normal vs. tumor adipose tissue, representing usage of alternate promoters in exon I (i.e., promoters I.3, II and I.4) (Agarwal et al., 1996; Zhou et al., 2001). Normal breast adipose tissue contains only low levels of aromatase and utilizes distal promoter I.4, whereas tumor adipose tissue expresses aromatase at higher levels and its mRNA is transcribed off proximal promoters II and I.3 (Zhou et al., 2001). Also, terminal differentiation of fibroblasts into mature adipocytes is accompanied by promoter-switching and greatly reduced aromatase expression. The underlying mechanism(s) of promoter switching are not well understood and the biological advantage(s), if any, conferred onto malignant cells remain undefined.

Body

A murine cell-culture (3T3-L1) model of fibroblast-to-adipocyte differentiation was validated and used to demonstrate aromatase gene promoter-switching. Validation of a human cell culture model to be used in further studies is nearly complete. The pace of progress was adversely affected by the loss of a technician who departed in February 2004 to relocate out of state with her family. Efforts to find a suitably qualified replacement were unsuccessful until October 2004. Completion of work proposed under Specific Aim 1, and Aims 2 and 3 (corresponding to Tasks 1, 2 and 3, respectively) during the current no-cost extension period will be greatly facilitated by the addition of a technician who has already been engaged to start work on November 29, 2004. Research accomplishments associated with each component of Task 1 are presented below: [Task 1: To examine aromatase mRNA translational efficiency associated with aromatase exon I alternate promoters I.3, II and I.4. a) Amplify and quantify invariant region encoded by exons II through X to enable measurement of total aromatase mRNA levels by competitive RT-PCR. b) Amplify individual aromatase species using promoter-specific primer sets and quantify each species by competitive RT-PCR, and c) Determine promoter-specific aromatase mRNA distribution in monosomes vs. polysomes and establish translational efficiency associated with aromatase exon I alternate promoters I.3, II and I.4.]

Task 1a and 1b): Human aromatase cDNA mimics for subsequent use in quantitative RT-PCR were constructed using the method described by Callaci and Hentosh (1997). We slightly modified and improved the protocol. Briefly, a heterologous "neutral" double-stranded DNA fragment of known sequence (Clontech) was PCR amplified using a pair of forward and reverse "composite" primers. The composite forward primer contained at its 5'-end, three sets of gene-specific (i.e., aromatase) nucleotides and the 20-mer forward primer (5'-CGTGACCCTCCCCGCTATCT-3') for the "neutral" double-stranded DNA. Oligonucleotide forward primers specific for aromatase promoters I.4, I.3, or PII were those described by Harada et al., (1993) as follows: promoter I.4 = 5'-GACCAACTGGAGCCTG -3'; promoter I.3 = 5'-CCTTGTT TTGACTTGTAAC-3'; promoter PII= 5'-AACAGGAGCTATAGATG-3'. They

were combined in the primer sequence: 5'-Promoter I.3-, PII-, and I.2-specific, followed by the 20-mer forward primer for the "neutral" double-stranded DNA (i.e., 5'-CCTTGTTTTGACTTGTAACAACAGGAGCTATAGATGGACCAACTGGAGCCTGCGTGA CC CTCCC CGCTATCT-3' The reverse primer for all the above mRNA species (and mimic) was derived from exon II: 5'-GTGCCCTCATAATTCCACAC-3'. Constructing one mimic incorporating all three primers specific for aromatase promoters I.4, I.3, and PII proved more expeditious than the originally proposed approach of making three separate constructs. Therefore this modified approach is being used in all subsequent experiments. A separate cDNA mimic was also constructed to amplify total aromatase transcripts, using the primer set described by Zhou et al., (2001) and "neutral" DNA specific nucleotides. All primers were synthesized and gel purified commercially (IDT Inc.) and subjected to one round of standard PCR using neutral DNA and composite primers. A second round of PCR was carried out using primers containing only aromatase-specific sequences. The resulting cDNA mimic was purified by centrifugation through Chromaspin® columns and then quantified by UV spectrophotometry.

Aromatase promoter usage was determined in three sets of 3T3-L1 cells: The first set of cells was untreated pre-confluent undifferentiated, the second was untreated spontaneously differentiated, and the third set was dexamethasone (DEX)-treated differentiated. A GenBank® search revealed that our human primers for promoters of interest (i.e., I.4, I.3 and PII) could be used in mouse cells. However, the reverse primer for the full human aromatase gene had insufficient homology with the mouse gene and could not be used in mouse cells. RNA was extracted from $5x10^6$ 3T3-L1 cells from each of the following three groups: preconfluent undifferentiated, spontaneously differentiated, and dexamethasone-induced differentiated cells. Briefly, cells were centrifuged for 10 min at 1100 rpm and the supernatant was aspirated. Cells were lysed using TRIzol (Gibco) and then chloroform was added to extract RNA, which was subsequently precipitated using isopropanol. RNA was washed with 75% ethanol and then pelleted and allowed to air-dry for 5-10 min. Thereafter, RNA was resuspended in nuclease-free water, allowed to incubate at 55-60°C for 10 min and then stored at -80°C while awaiting RT-PCR. mRNA (1.0 µg) from preconfluent undifferentiated, spontaneously differentiated, and dexamethasone-induced differentiated 3T3-L1 cells was reverse transcribed into cDNA in 1x PCR buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 1 mM each of 4 dNTPs, 1 unit/µl RNasin inhibitor, 2.5 µM random hexamer primers, and 2.5 units/µl Moloney Leukemia Virus reverse transcriptase (**MuLV-RT**). Mixtures were annealed at room temperature for 10 min, incubated at 42°C for 40 min, heated at 95°C to inactivate MuLV-RT, and cooled for 5 min. Samples were amplified with promoter specific primers for 35 cycles (94°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec) with a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose/Tris-borate-EDTA gel (Fig. 1). We suspect that the band visible at ~100 bp in lanes 4, 5, 8, 9 and 13 resulted from nonspecific primer annealing and amplification.

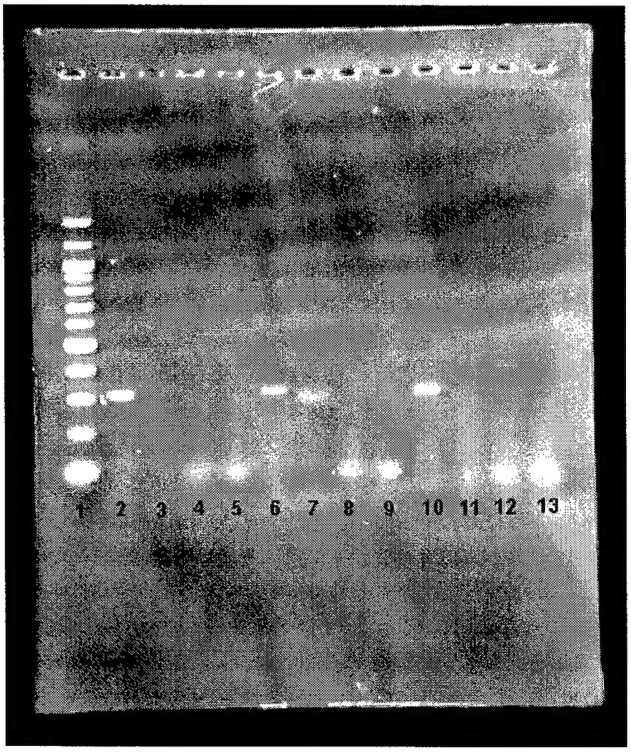

Fig. 1

Lane 1: 100 bp molecular weight marker

Lanes 2-5: Spontaneously differentiated 3T3-L1 cells
Lane 2: Promoter 1.3- specific aromatase mRNA
Lane 3: Promoter PII-specific aromatase mRNA
Lane 4: Promoter 1.4-specific aromatase mRNA
Lane 5: -RT

Lanes 6-9: Preconfluent undifferentiated 3T3-L1 cells
Lane 6: Promoter 1.3- specific aromatase mRNA
Lane 7: Promoter PII-specific aromatase mRNA
Lane 8: Promoter 1.4-specific aromatase mRNA
Lane 9: -RT

Lanes 10-13: DEX-treated differentiated 3T3-L1 cells
Lane 10: Promoter 1.3- specific aromatase mRNA
Lane 11: Promoter PII-specific aromatase mRNA
Lane 12: Promoter 1.4-specific aromatase mRNA
Lane 13: -RT

Preconfluent undifferentiated 3T3-L1 cells (lanes 6-9) expressed both promoter 1.3- and promoter II-specific aromatase mRNA. A similar pattern of promoter usage was reported in breast cancer tissue (Bulun et al., 1997; Harada, 1997; Zhou et al., 1996). Spontaneously differentiated cells (lanes 2-5) expressed only promoter 1.3-specific aromatase mRNA. In contrast, DEX-treated differentiated 3T3-L1 cells (lanes 10-13) expressed both promoter 1.3- and I.4-specific aromatase mRNA. Interestingly, with the I.4 specific primer set, two faint bands were observed in lane 12 (MW ~250 and ~400, respectively). Previous reports (Harada, 1992; Mahendroo et al., 1993) indicated that promoter I.4 was predominant in human adipose stromal cells and fibroblasts from normal breast tissue. Our preliminary findings suggest that spontaneously differentiated 3T3-L1 cells may not be equivalent to DEX-treated differentiated cells when used to investigate promoter-switching associated with breast tumorigenesis. More importantly, the possibility of biochemical heterogeneity and any attendant importance in human cell culture systems subjected to selected differentiation treatments will be established to insure increased applicability of future findings.
Task 1c): Currently in progress with completion projected towards the end of January 2005.

Key Research Accomplishments

- Design, construction and validation of cDNA mimics.
- Design, construction and validation of primer-specific aromatase mRNA variants.
    - Amplification of invariant region encoded by exons II through X.
    - Amplification of individual aromatase species.
- Establishment and validation of murine cell-differentiation protocols as a template for human breast cell-differentiation protocols currently undergoing validation.

Reportable Outcomes

- The following reportable outcomes are expected beginning in the first quarter of 2005:
    - One or more manuscripts
    - One or more abstracts and presentation(s).
    - One or more applications for funding from a national granting-making agency.

Conclusions

Preliminary findings have confirmed the technical feasibility and soundness of key aspects of the proposed work. It is anticipated that interesting and useful information will be obtained from current studies that could provide a strong foundation for more substantial future grant applications. We would therefore not request any changes in the approved statement of work at this time.

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Appendices

None